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STABILITY, INHIBITION AND REACTIVATION OF ACETYLCHOLINESTERASE COVALENTLY COUPLED TO GLASS

GEORGE BAUM, FRANK B. WARD AND HOWARD H. WEETALL
Research and Development Laboratories, Corning Glass Works, Corning, N.Y. 14830 (U.S.A.)

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SUMMARY

Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) was covalently coupled to a silanized fritted glass disc by a diazonium salt reaction and by a diimide condensation. The diimide-coupled adduct survived numerous assays and chemical studies for a period of 55 days at room temperature in contrast to the diazonium-coupled adduct which rapidly lost activity. Both adducts exhibited a lesser pronounced dependence of activity on pH than the free enzyme, reflecting the weak buffering action of the silanol–silane surface. The diimide adduct is inhibited by diethyl *p*-nitrophenyl phosphate and can be reactivated by either Toxigonin or alkaline phosphatase (orthophosphoric monoester phosphohydralase, EC 3.1.3.1).

INTRODUCTION

In the last few years workers at our laboratory have investigated inorganic materials for the direct adsorption of enzymes[1] and as a supporting structure for the covalent bonding of enzymes through reactive siloxane polymers[2–5]. Inorganic supports are dimensionally stable, relatively inert, and the bound enzyme is less structurally shielded than when it is entrapped within a polymer matrix. Cholinesterases have been immobilized in starch gels by Bauman *et al.*[7] and by Guilbault and Das[8]; in polyacrylamide gels by Guilbault and Das[8] and by Degani and Miron[9]; in a silastic resin by Pennington *et al.*[10] and covalently coupled to Sepharose[11]. The gels are difficult to handle and support. Entrapment in silastic resin appeared to give a less-stable product. We now report our results on the preparation and characterization of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) covalently coupled to a fritted glass disc. The discs are readily available and are easily transferred between reagent solutions.

MATERIALS AND METHODS

Silanization

All discs employed in this study were Corning No. 3100, medium porosity, 2.0 cm in diameter with a plain edge. The discs were cleaned in hot 4 M HNO_3,

washed well with distilled water, and baked dry at 400 °C for 12 h. The discs were then silanized with γ-aminopropyltriethoxysilane (Union Carbide, A-1100) in refluxing toluene (1% solution) for 3 h. Excess silane was removed by thorough washing with methanol.

Diimide coupling

Each disc was placed in 5 ml of phosphate buffer solution maintained at pH 4.0 and exhaustively degassed by repeated evacuation. 5 mg of acetylcholinesterase, E. electricus, (Worthington Biochem., 130 I.U./mg) and 5 mg of 1-cyclohexyl-3 (2-morpholinoethyl) carbodiimide, metho-*p*-toluenesulfate (Mann Res.) were added. The solutions were placed in screw cap jars and stored at 5 °C for 16 h. The discs were rigorously flushed with 1.0 M saline solution, distilled water, and then subjected to repeated assay.

Diazo coupling

The reactions for the preparation of adiazo-coupled enzyme have been previously described[3]. The aminosilane-treated glass is reacted with *p*-nitrobenzoic acid and the resulting nitrobenzamide is reduced and diazotized.

Assay

All assays were conducted using the acetylcholine-selective electrode procedure[12]. Acetylcholine bromide at $2 \cdot 10^{-3}$ M concentration in phosphate buffer was used as the substrate solution. The rate of change of acetylcholine concentration from $1.95 \cdot 10^{-3}$ M to $1.90 \cdot 10^{-3}$ M was determined by monitoring the potential across a CGW 476200 acetylcholine-selective electrode and a Fisher 13-639-57 cracked bead calomel reference electrode with a digital electrometer (Corning Model 101). Assays were conducted at 25 °C ± 0.02 °C.

Reactivation

Toxigonin [oxybis-(4-hydroxyiminoethyl pyridinium-1-methyl) dichloride] was obtained from A. G. Darmstadt-Merck. A 0.10% solution in phosphate buffer pH 8.0 was used for reactivation. The alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) (bacterial) was obtained from Worthington Biochemical. The discs were immersed in 10 ml phosphate buffer solution, pH 8.0, and 1 ml (350 units) of phosphatase was added.

RESULTS AND DISCUSSION

Coupling reactions and stability

Shortly after the diazo coupling reaction, the discs exhibit a relatively high activity which decreases rapidly on repeated washing and usage. Only about 0.5% of the activity of the enzyme reactant solution appears on the discs. Since we use a relatively crude acetylcholinesterase, nonspecific coupling to concomitant proteins undoubtedly occurs. Adsorption of protein without reaction does occur and the adsorbed acetylcholinesterase does retain activity. The diazo-coupled adducts rapidly lost activity. In about 10 days the activity decreased from 0.15 unit per disc to 0.05 unit per disc (Fig. 1). The high initial activity values are attributed to adsorbed enzyme.

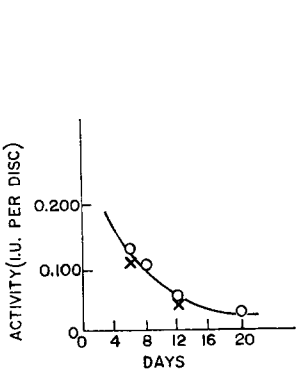


Fig. 1. Stability of acetylcholinesterase diazo coupled to two glass discs.

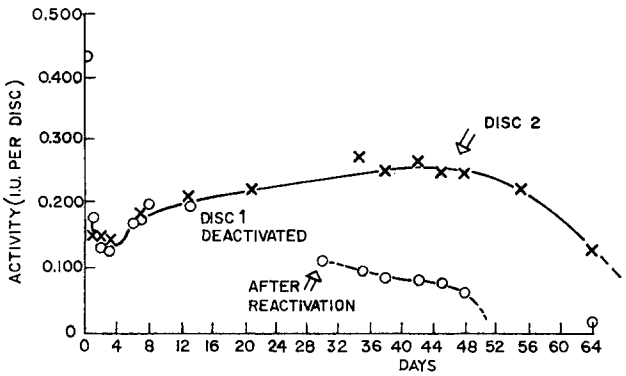


Fig. 2. Stability of acetylcholinesterase diimide coupled to glass discs.

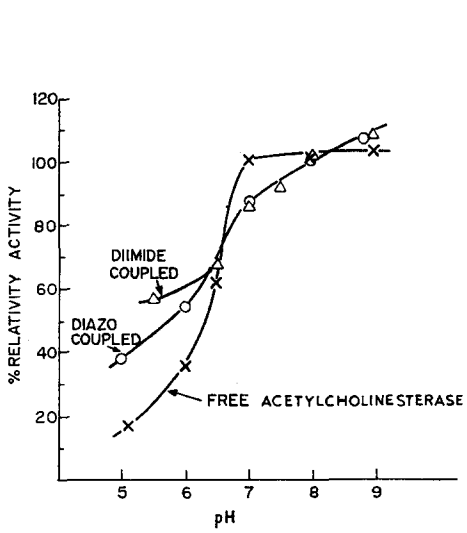
An alternative coupling reaction employed was the diimide-promoted amide formation[13] between the aminosilane-treated glass and free carboxylic acid groups of the enzyme. The diimide-coupled enzyme adducts exhibited significantly improved stability. After an initial leaching period of several days, the enzymic activity stabilized at about 0.25 unit per disc for 55 days before the activity decreased below convenient activity levels (Fig. 2). The discs were stored in phosphate buffer at room temperature between assays. If the discs were refrigerated, then they must be maintained at room temperature for several hours before a constant activity value can be obtained. Free acetylcholinesterase solutions at room temperature in our laboratory have a usable life of about 25 days before the activity falls below a convenient value of about 25% of the initial activity.

pH effects

The pH dependence of the activity of the enzyme adducts bound by the two processes are essentially identical and different from free acetylcholinesterase. Whereas the free enzyme rapidly increases in activity in the pH 6–7 range[14], the bound enzyme adducts increased gradually in activity and did not reach a maximum below pH 9.0. The data are shown in Fig. 3. Silman and Katchalski[14] have attributed differences in the pH–activity behavior between free and immobilized enzymes to charge effects within the microenvironment. A mild buffering action in the aminosilane–glass micro-environment is attributed above pH 7.0 to weakly acidic silanols at the glass surface and below pH 7.0 to unreacted primary amine groups of the γ-aminopropylsilane. We have observed a small alkaline shift (above pH 7.0) in the pH optimum of L-amino acid oxidase which was coupled to porous glass by the diazo procedure[5].

Inhibition and reactivation

A second diimide coupled acetylcholinesterase adduct was assayed at frequent intervals for twenty days and exhibited stability similar to disc 2 of Fig. 2. The enzymic activity of this disc was completely inhibited by treatment with paraoxon (diethyl *p*-nitrophenyl phosphate). The phosphorylated enzyme adduct can be reactivated by several different agents. Freshly inhibited discs can be reactivated by



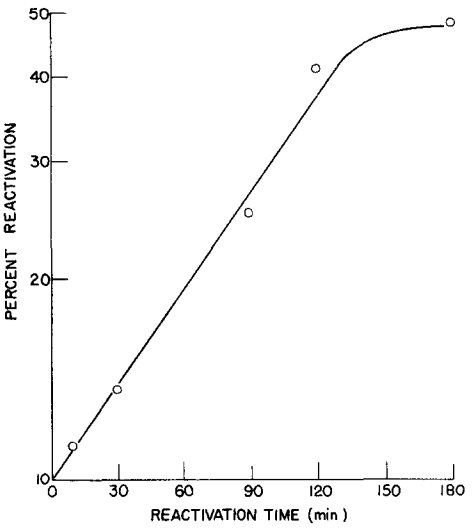


Fig. 3. pH dependence of the activity of bound acetylcholinesterase.

Fig. 4. Reactivation of phosphorylated immobilized acetylcholinesterase by alkaline phosphatase.

Toxigonin, as reported by Wilson for free acetylcholinesterase[16], to nearly the initial activity values. However, attempts to reactivate the phosphorylated enzyme adduct with Toxigonin after 24 h storage were only partly successful. About 70% of the initial activity of the discs can be reactivated.

In a separate experiment an aged phosphorylated acetylcholinesterase adduct was reactivated by alkaline phosphatase. The kinetics of the reactivation reaction was pseudo first order with respect to the amount of phosphorylated adduct present (Fig. 4). Only 40% reactivation could be accomplished. This disc continued to exhibit a relatively stable level of activity for an additional 18 days before the activity fell below a convenient assay level (Fig. 2).

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